5

Vacuum-Drying and Cryopreservation of Prokaryotes

Brian J. Tindall

Summary

Traditionally, the *ex situ* study of prokaryotes (members of Archaea and Bacteria) has required access to living cultures. Although it may be possible to maintain a small number of strains by serial transfer, such methods are not the best, particularly for long-term storage or for keeping a large collection. One of the major problems is dealing with the vast range of organisms that have been isolated to date. Particularly in the case of methods of cultivating prokaryotes, it is almost impossible to give a simple list of media to use. However, general principles are outlined that should cover most of the currently known prokaryotes in pure culture. The methodologies used for the long-term storage of prokaryotes may be divided into those that involve freezing and those that involve vacuum-drying. The methods described are those that are either used in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (http://www.dsmz.de/index.htm) or in other national culture collections.

Key Words: Prokaryotes; bacteria; archaea; freezing; freeze-drying; long-term storage.

1. Introduction

The long-term storage of strains is an issue that few scientists pay much attention to. Strictly speaking the reproducibility of every scientific publication that involves the use of a particular strain depends on the availability of that strain. In addition, databases such as EMBL/GenBank are plagued by the accumulation of sequences attributed to organisms that have been wrongly labeled, "mixed up," or contaminated. It is impossible to estimate how much of the published literature is affected by the nonavailability of strains to subsequent researchers either to check the identity of the strains concerned or to reproduce the results. Serial transfer of strains over long periods of time is not the best way of maintaining strains. Some form of

From: *Methods in Molecular Biology, vol. 368: Cryopreservation and Freeze-Drying Protocols, Second Edition*
Edited by: J. G. Day and G. N. Stacey © Humana Press Inc., Totowa, NJ

storage in a fashion that keeps the strain in an inert, but potentially viable state, is recommended.

Over the decades a number of methods have been developed to store strains. The choice of methods to be used often depends on the time period over which strains are to be maintained, as well as whether strains are to be shipped frequently. The goal of a research group may be to maintain strains during the duration of a 5-yr research project, whereas a national culture collection is interested in storing the strains for decades. In some cases, it may be wise to use two different methods, one for working stocks and one for long-term "back ups." Storage in liquid nitrogen, if available, is probably a fairly good "all round" method. In particular, the capillary method currently used in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) has a number of advantages. Storage on beads or in glycerol in mechanical freezers may be more suitable for maintaining strains for the duration of research projects.

One cannot stress enough the importance of not only the proper storage of strains long term, but the fact that viability of stored batches must be checked at the time they are prepared, as well as some form of authenticity/identity check. The largest national collections check the viability of all stocks are they are prepared, which comes as a surprise to many customers when a strain fails to subculture in their laboratory. In many cases, long-term storage of anywhere between 5–35 yr being documented for freeze-dried cultures ***(1)***. Data on the longevity of storage in liquid nitrogen is still missing, simply because the method has only been comparatively recently introduced. Experience in the DSMZ (founded in 1969) indicates that the vast majority of the original stocks have survived 30 yr.

Although it is the goal of the present chapter to give detailed methods for the storage of prokaryotes, with the exception of cyanobacteria (*see* Chapter 10), each laboratory will find that some of the consumables used will vary slightly, and that the equipment used will depend on the local suppliers. The second greatest variable is the sheer diversity of prokaryotes that are being isolated. The DSMZ currently has a list of well over 1000 different media that are required for their holdings and will certainly continue to grow. Some methods of storing strains can be applied to a wide variety of strains, but there will always be one or two strains that may behave differently. Sometimes the literature may emphatically declare that a particular group of organisms cannot be frozen and/or dried. However, adapting standard protocols and studying the mechanisms organisms use to survive various forms of "dehydration" may help to overcome some of these problems. The present chapter attempts to bridge the gap between some general principles and some detailed protocols. Apart from

Fig. 1. Glass centrifuge tubes made by Ochs (Bovenden, Germany). To the left, a glass centrifuge tube used for aerobic strains. To the right, a centrifuge tube used for anaerobic strains. The narrow neck is sealed with a rubber stopper, which fits inside the screw cap, and has a hole in the top.

hands-on experience with a variety of methods, two books have been particularly useful, Lapage and Redway ***(2)*** and Kirsop and Doyle ***(3)***.

2. Materials

2.1. Standard Materials Used for All Techniques

1. Laminar flow cabinet and appropriate facilities for following good microbiological practice.
2. All glassware should be sterilized, and all media and suspending liquids sterilized by filtration or autoclaving as appropriate.
3. Use a liquid culture or agar slope culture grown to the late log or early stationary phase.
4. Custom-made glass centrifuge tubes (Ochs, Bovenden, Germany) in which liquid cultures of strains may be grown and harvested (**Fig. 1**).

2.2. Cryopreservation Methods

2.2.1. Cryopreservation in Glycerol

1. Glycerol. This is best dispensed if it is warmed gently to allow it to flow better.
2. Sterilized glass screw-cap bottles. 15 mm (outside diameter) × 50-mm bottles are used in the DSMZ. The bottles should not be too small and should be easy to handle and open without the risk of contamination.

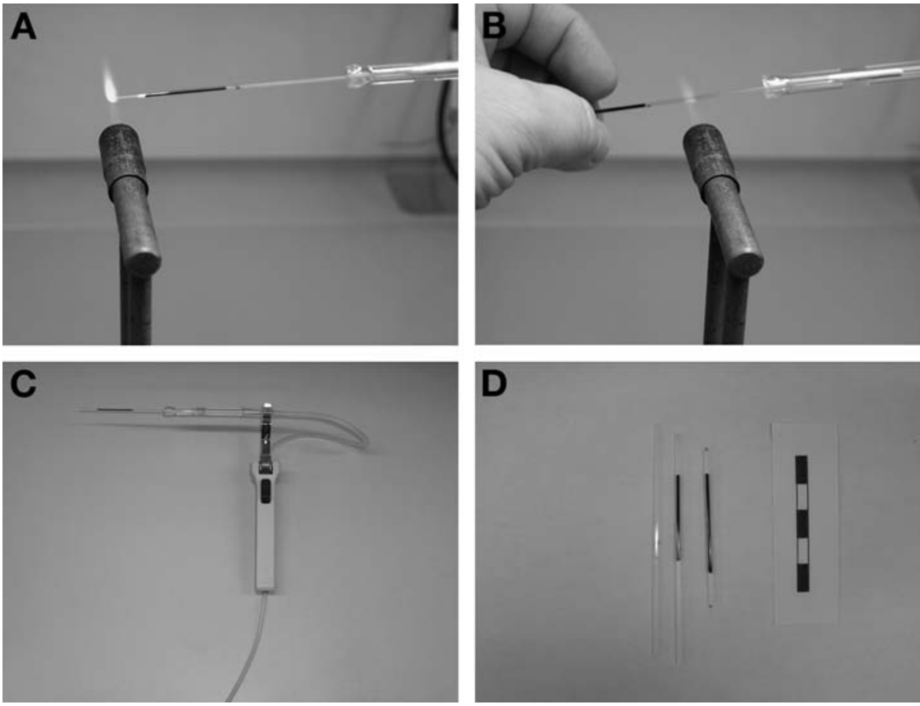

Fig. 2. The process of making a glass capillary. **(A)** Heating the open end of the capillary to seal it. Note the fine hot flame that only comes into contact with the very tip of the capillary. **(B)** Once the open end is sealed the capillary may be cut from the adapter by heating about 25–30 mm from the top end (to the right). Note the use of the fine hot flame, which only comes into contact with a very limited length of the capillary. **(C)** The capillary held in the glass adapter, which is fitted to the "microclassic" (Brand) pipetting aid. The region where the capillary is filled has been made visible with dyed water (capillary to the left). **(D)** Capillaries. To the left, empty, in the middle filled (top toward the adapter), right sealed vials.

2.2.2. Cryopreservation on Glass of Ceramic Beads

1. Small glass or ceramic beads (in different colors) can be obtained from a variety of sources, ranging from hobby shops to those selling embroidery products. Two to three millimeter external diameter is the ideal size, but care should be taken that larger sizes do not hinder their removal from the glass bottle.
2. Screw-capped glass bottles, about 10–15 mm (outside diameter) × 30–50 mm.

2.2.3. Cryopreservation in Glass Capillaries

1. Glass capillaries 1.4 mm (outside diameter) × 90 mm, wall thickness 0.261 mm (article no. 1400290 from Hilgenberg GmbH, Malsfeld, Germany) (**Fig. 2D**).
2. Micropipetting aid (microclassic from Brand) (**Fig. 2C**).

3. A custom-made adapter for fitting into the micropipetting aid in order to hold the capillary (*see* **Note 1**).
4. Aluminium miniature pipet canisters are used to store and sterilize the glass capillaries (top section approx 18 × 40 mm, lower section, which slides into the upper section, approx 16.5 × 80 mm). Usually 20 or 40 are placed in the canister at one time.
5. 11 mm × 43.5-mm small glass vial fitted with a cotton plug (the type of plug used in the DSMZ can be obtained from suppliers of dental consumables) (*see* **Fig. 3B**,**C**). This is sterilized and used to hold the concentrated cell suspension. In practice, a number of such vials are sterilized by placing them in a glass beaker of suitable size, covering the mouth of the beaker with a lint cover, and covering that with aluminium foil. The beaker is then autoclaved.
6. An ice bath.
7. A glass Petri dish.
8. A 10-mL glass, screw-capped test tube filled with ethanol and cooled on ice.
9. Paper tissue for drying the finished capillaries.
10. A gas burner that produces a fine hot flame that can be adjusted. Some natural gas-compressed air miniature torches may be suitable. Soldering torches, such as those manufactured by Camping Gaz, are also suitable.
11. Cryoprotectant: glycerol or dimethyl sulfoxide (DMSO) may be used. The final concentration of glycerol is usually 10–15% (v/v). DMSO is used at a final concentration of 5% (v/v). DSMO may be either filter sterilized or autoclaved under a nitrogen atmosphere at 115°C for 15 min.

2.3. Drying/Freeze-Drying Methods

2.3.1. Materials Needed for All Methods

1. Constrictor (*see* **Note 2**).
2. Freeze-drier (*see* **Note 3**).
3. Gas burner for sealing the ampoules.

2.3.2. Cryopreservation and Drying or Drying From a Predried Plug

1. –20°C Freezer.
2. 20% (w/v) skimmed milk, sterilized by autoclaving at 115°C for 13 min. 5 mL will produce 25 ampoules. The skimmed milk is best sterilized in 5-mL portions in glass screw-capped tubes.
3. A Pasteur pipet may be used, but sterile multistepper pipetting aids such as those made by Eppendorf are more accurate and easier to handle. A multistepper delivering 200 μL is used in the DSMZ.
4. 11 mm × 43.5-mm small glass vials, fitted with a cotton plug (the type of plug used in the DSMZ can be obtained from suppliers of dental consumables) (**Fig. 4B**).
5. Suitable material for labeling the outside of the inner vial.
6. 14.5 mm × 100-mm soda glass tubes, into which has been placed a few pellets of a silica-based humidity indicator and a layer of cotton wool (**Fig. 4B**).

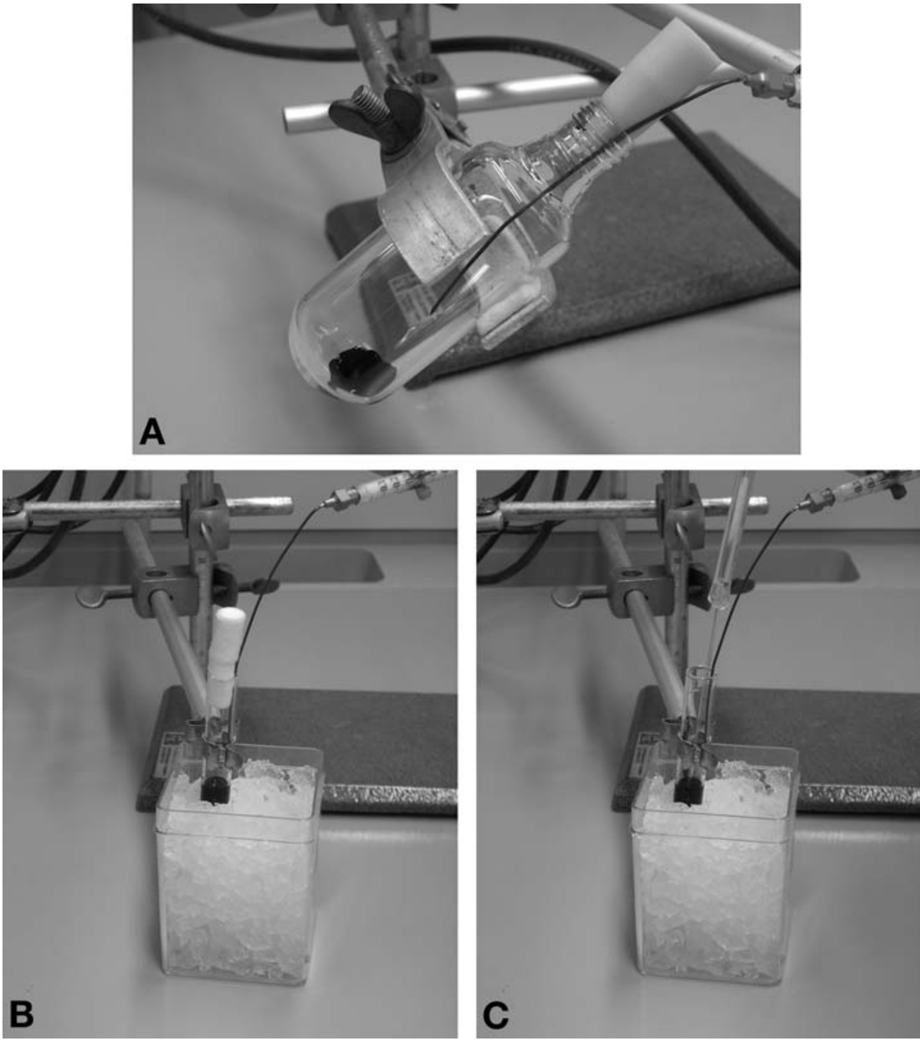

Fig. 3. The process of handling anaerobic or oxygen-sensitive prokaryotes for capillary freezing. **(A)** The centrifuge tube with the rubber stopper removed and replaced by a soft silicon stopper. The supernatant has been removed and the cell suspension is gassed via a sterile syringe needle (that enters the tube from the right and extends to just above the cell suspension). **(B)** The cell suspension is transferred to a small (sterile) vial, fitted with a cotton stopper. The vial is gassed with a sterile syringe needle (that enters from above). The vial is stored on ice. **(C)** The cell suspension is drawn into the capillary using the adapter and pipetting aid. The cell suspension is gassed continually with a flow of sterile nitrogen.

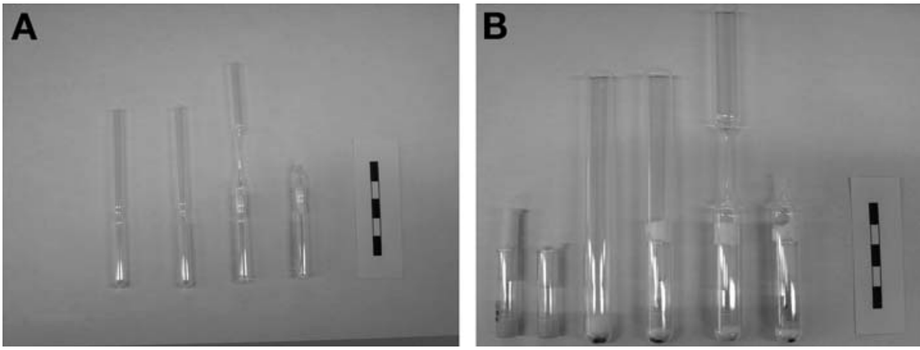

Fig. 4. Processes in the making of freeze-dried vials. **(A)** 7–8 × 100-mm vials. (Left to right) The original vial, a vial fitted with a cotton plug, a constricted vial, and a finished vial. **(B)** Double-vial system used in the DSMZ. (Left to right) The inner vial containing sterile, freeze-dried skimmed milk, the vial with the cotton stopper shortened ready to be placed in the outer tube, the outer tube with a few crystals of a silica gel-based humidity indicator and a layer of cotton-wool, the inner vial packed into the outer tube held in place by a glass wool plug, the constricted ampoule, and the finished ampoule.

7. Glass wool (depending on the product this may cause respiratory problems or other health hazards if inhaled—take appropriate precautions) and a plastic "ramrod" that fits loosely into the soda glass tubes.

2.3.3. Centrifugal Drying

1. "Mist desiccans."
   a. 100 mL horse serum.
   b. 33 mL oxoid nutrient broth (CM1).
   c. Mix the two solutions and add 10 g glucose.
   d. Dissolve the glucose by slow, steady shaking.
   e. Sterilize by over pressure filtration through a membrane filter.
2. Blotting paper, bearing the strain designation and date of preparation of the ampoule.
3. Freeze-drier (fitted with a centrifuge) (*see* **Note 3**).
4. Gas burner for sealing the ampoules.
5. Sterile Pasteur pipets.
6. 7 mm × 100-mm glass vials. Plug with cotton wool, sterilize in covered racks or containers, with the labels (**Fig. 4A**).

3. Methods

3.1. Cultivation of Strains Prior to Preservation

Each strain to be either stored frozen or in a dried state should be cultivated on the medium on/in which it grows best. The list of possible media and growth

conditions is far too extensive to be dealt with here but the following sections deal with some general principles.

3.1.1. Cultivation of Aerobes

Aerobic strains are usually grown either on agar slopes or in liquid media. It is impossible to generalize as to how many agar slopes or what volume of liquid culture is to be prepared, and one has to rely on experience. Generally either three to five agar slopes (in 16 mm × 180-mm test tubes) or about 20 mL of liquid culture are inoculated and used as the source of cell material for the preparation of a concentrated cell suspension. Some strains grow under special gas mixtures, such as hydrogen-oxidizing bacteria or methanotrophs (*see* **Note 4**).

Organisms that are difficult to remove from the agar surface may be better cultivated in liquid culture. Some organisms, such as filament-forming actinomycetes, may form aggregates in liquid culture, which may be difficult to break up under normal conditions. Strains should be grown under optimal conditions and harvested in the late exponential/early stationary phase. In the case of organisms that produce spores, they should be grown under conditions that enhance the numbers of spores produced.

3.1.2. Cultivation of Oxygen-Sensitive Organisms

Some microaerophiles or anaerobes may be grown on agar slopes in tubes that are filled with the appropriate gas mixtures and sealed with appropriate rubber stoppers ***(4)***. However, in laboratories, such as the DSMZ, where the Hungate technique ***(5)*** is a routine method, it has been found more convenient to grow such strains in liquid culture. It is normally necessary to harvest cells by centrifugation and it may be easier to cultivate the strains directly in suitable, custom-made glass centrifuge tubes, fitted with a screw cap and a suitable, gas-tight rubber stopper. Strains should be grown under optimal conditions and harvested in the late exponential/early stationary phase. In the case of organisms that produce spores, they should be grown under conditions that enhance the numbers of spores produced.

3.1.3. Harvesting Aerobic/Oxygen-Tolerant Strains From Agar Slopes

1. Cells that can be easily removed from the agar are removed from the surface by repeated gentle washing with a small volume of the appropriate suspending solution (usually about 1–5 mL) (*see* **Note 5**).
2. Because of the different methods that can be used for either freezing or drying cells, the suspending medium may be either fresh growth medium, fresh growth medium containing cryoprotectants (for freezing only), skimmed milk, or other suitable protective agents (*see* **Note 6**).

3.1.4. Harvesting Aerobic/Oxygen-Tolerant Strains by Centrifugation

1. Oxygen-tolerant strains may be either grown in separate vessels (e.g., conical flasks or serum bottles for those requiring special gas mixtures) or the organisms may be grown directly in the appropriate glass centrifuge tubes. Custom-made glass centrifuge tubes (Ochs) in which the strains may be grown and harvested, or the liquid cultures are transferred to them in order to be harvested are illustrated in **Fig. 1** (*see* **Note 7**).
2. Cells are generally harvested in a swinging bucket rotor, running at about 3000 rpm for 20 min (*see* **Notes 8** and **9**).
3. The rotor should be braked at a speed that will not cause the sedimented cell pellet to resuspend.
4. The supernatant is carefully removed using a suitably sized pipet and the remaining cell pellet resuspended in the appropriate medium (*see* **Note 6**).

3.1.5. Harvesting Anaerobic/Oxygen-Sensitive Strains From Agar Slopes

This method is included for completeness, but such organisms are generally not grown on slopes in the DSMZ. All manipulations during the process of harvesting must be carried out under the appropriate gas mixture. Cells that can be easily removed from the agar are removed from the surface by repeated gentle washing with a small volume of the appropriate suspending solution (usually about 1–5 mL) (*see* **Notes 5** and **6**).

3.1.6. Harvesting Anaerobic/Oxygen-Sensitive Strains by Centrifugation

1. Oxygen-sensitive or anaerobic strains may be either grown in separate vessels (e.g., serum bottles), or the organisms may be grown directly in the appropriate glass centrifuge tubes (*see* **Note 10**).
2. All manipulations must be carried out under oxygen-free (or limiting) conditions (*see* **Note 7**).
3. Cells are generally harvested in a swinging bucket rotor, running at about 3000 rpm for 20 min (*see* **Notes 8** and **9**).
4. The rotor should be braked at a speed that will not cause the sedimented cell pellet to resuspend.
5. The supernatant is carefully removed (under an appropriate gas atmosphere) using a suitably sized pipet and the remaining cell pellet resuspended in the appropriate medium (*see* **Note 6**).

If precautions are not taken then no survival will be observed after "reviving" preserved material. Certain organisms may be difficult to manipulate, grow, and subsequently preserve if not dealt with in the proper fashion (*see* **Notes 11–15**).

3.2. Cryopreservation-Based Preservation/Stabilization Techniques

3.2.1. Storage by Cryopreservation in Mechanical Freezers

3.2.1.1. Cryopreservation in Glycerol

1. Dispense the glycerol in 1-mL portions into the glass bottles and sterilize in an autoclave.
2. Once cooled, the glass bottles may be labeled on the sides or by painting the top of the cap with Tippex and writing the strain designation with a fine, water-insoluble, felt-tipped pen.
   a. To prepare the strain for storage remove 0.6 mL of the grown culture and add it to the glycerol; mix gently to give a homogeneous solution.
3. Add a freshly grown liquid culture to sterilized glycerol in appropriate screw-capped glass bottles.
4. Freeze the bottles at –20°C and store in a conventional freezer.
5. When samples are withdrawn, it is not necessary to thaw the bottles and they may be conveniently held in a precooled wax block containing appropriate sized holes. It should be noted that every time that a sample is withdrawn that there is always a chance of contamination.

3.2.1.2. Freezing Using Glass/Ceramic Beads

This method is essentially that developed by Feltham et al. ***(7)*** and may be used to store organisms either in mechanical freezers (–20 to –80°C), or using liquid nitrogen (*see* **Note 16**). If commercially available systems are to be employed then one simply follows the manufacturer's instructions.

1. Wash the beads in detergent, followed by dilute HCl (to neutralize the alkalinity of the detergent). The beads are then washed several times with tap water, and finally with distilled water. The beads are then dried at 40–50°C.
2. Add 10–20 beads to the glass bottles, loosely fit the caps, and sterilize them. Different colored beads may be used to distinguish different groups/categories of organisms.
3. Cell suspensions may be obtained either by washing the strains from agar slopes, or by centrifugation of liquid cultures.
4. Resuspend the culture in the same growth medium (in broth form) containing 15% (v/v) glycerol (*see* **Note 17**).
5. Pipet the cell suspension into the glass vials, using a Pasteur pipet, so that the beads are fully covered.
6. Introduce the end of the pipet to the bottom of the vial and use the pipet to empty and recover the beads several times. Gently agitate the beads to dislodge any air bubbles. Once the beads are fully wetted, the vial is held at a slight angle and the excess cell suspension removed from the lowest point of the bottom of the vial. Excess liquid will only cause the beads to stick together.
7. Place the vials in the freezer (usually –60 to –80°C).

The same method may be used for the storage of strains in liquid nitrogen. However, it is generally recommended that plastic, screw-cap vials are used and that they be stored in the gas phase. In addition, the approach may be used for the storage of anaerobes (*see* **Note 18**).

3.2.2. Storage Above or in Liquid Nitrogen (see ***Notes 19–21****)*

3.2.2.1. Freezing in Glass Capillaries

The method described here is that which was developed in the DSMZ during the 1970s in order to cope with the growing number of "exotic" strains that were being deposited, and is an adaptation of the method described by Hippe ***(5)*** for anaerobes.

1. DMSO is generally added to freshly prepared, sterile growth medium to give a final concentration of 5% (v/v). DMSO is always added just before the medium is used, and the mixture is never stored. Cool the medium containing DMSO in an ice bath.
2. Harvesting the cells. Use strains grown to the late exponential to early stationary phase.
   a. If grown on slopes the cells are washed off using about 1 mL of fresh, sterile medium containing 5% DMSO. If several slopes are used then the washing medium, containing an increasing concentration of cells, is transferred from tube to tube. The final, concentrated cell suspension is then transferred to the small, cotton-plugged vial and placed on ice.
   b. If cells are harvested by centrifugation of liquid media, then the supernatant is carefully removed and the cell pellet resuspended in 1 mL of fresh, sterile medium containing 5% (v/v) DMSO. In some cases it is not easy to obtain a compact pellet and in such cases the cells may be diluted 1:1 with fresh sterile medium containing 10% (v/v) DMSO (the final concentration of DMSO is 5% v/v).
3. The final, concentrated cell suspension is then transferred to the small, cotton-plugged vial and placed on ice.
4. Place the adapter in the pipetting aid using normal microbiological techniques to avoid contaminating the end into which the capillary will be inserted.
5. Insert a capillary (under aseptic conditions) into the adapter (**Fig. 2C**).
6. Remove the cotton plug and dip the tip of the capillary into the concentrated cell suspension held in the small vial and slowly withdraw sufficient cell suspension to fill the capillary to about 40 mm from the lower end (**Fig. 3C**). Remove the end of the capillary from the cell suspension and gently draw the cell suspension further up the capillary so that the lower end of the cell suspension is about 10 mm from the lower (open) end. Remove the capillary from the vial and replace the cotton stopper.
7. Place the open end of the capillary in the fine gas flame, just above the hottest part of the flame (**Fig. 2A**). The end of the capillary should melt very quickly and seal in a slightly rounded drop. Holding the capillary too long in the flame will only

heat the culture and kill the suspension. Excessive heating of the end may also cause the gas held between the closed end and the suspension to expand, causing the end to form a thin-walled glass ball—this is fragile and will usually break.

8. Remove the end of the capillary from the flame and place the region between the upper surface of the cell suspension and the adapter about 25–30 mm from the upper end of the capillary in the tip of the flame (**Fig. 2B**). The glass should melt and collapse, sealing the capillary, and at this point the capillary is separated by pulling the two ends apart. The capillary is usually about 60-mm long, with a 40-mm long cell suspension 10 mm from each end.
9. Place the sealed capillary in the glass Petri dish that is kept in the ice bath. Remove the short end of the capillary that is still held in the adapter with forceps. (**Warning**: the end will still be hot.)
10. Repeat **steps 1–9** until sufficient capillaries have been made (*see* **Note 22**).
11. The finished capillaries may be immersed in the cold alcohol to sterilize the outside. All capillaries are then dried, by rubbing them gently between absorbent tissue paper, and placed in their storage container (*see* **Fig. 5A**).
12. The capillaries, in their storage container are then placed in a labeled plastic Petri dish and laid in the gas phase of the liquid nitrogen tank. Once frozen, the capillaries in their storage container may be placed in their storage position (*see* **Notes 23** and **24**).

The method may also be used to store anaerobic or oxygen sensitive-strains in capillaries in liquid nitrogen. The methods used in the DSMZ for the storage of anaerobes or oxygen-sensitive organisms generally uses the Hungate technique for handling the strains ***(5)***, which employs additional precautions to ensure that the strains are not exposed to oxygen.

1. Strains are either cultivated in suitable anaerobic containers or directly in specially made centrifuge tubes, which may be maintained under the appropriate gas mixture.
2. Harvest the cells by centrifugation; remove the supernatant under a stream of nitrogen gas that is led into the tube via a long, sterile (sterilized by flaming) syringe needle. The gas should be passed through a sterile cotton plug to prevent contamination from the nitrogen gas lines (**Fig. 3A**).
3. Resuspend the cell pellet in anaerobic medium containing DSMO, which is added just before use. The points mentioned in **Subheading 2.2.** are to be followed, remembering that all work is to be carried out under a flow of sterile nitrogen gas (**Fig. 3B**,**C**). The final concentrated cell suspension is then transferred to the small, cotton-plugged vial, gassed with nitrogen, and placed on ice (**Fig. 3B**,**C**) (*see* **Note 25**).
4. Follow **steps 4–12**, with the exception that in **step 6** the tip of the capillary is first held above the cell suspension, and nitrogen is drawn into the capillary and adapter before the capillary is filled with the cell suspension and sealed out outline in the subsequent steps.

This method is routinely used in the DSMZ for storing some of the most fastidious anaerobes.

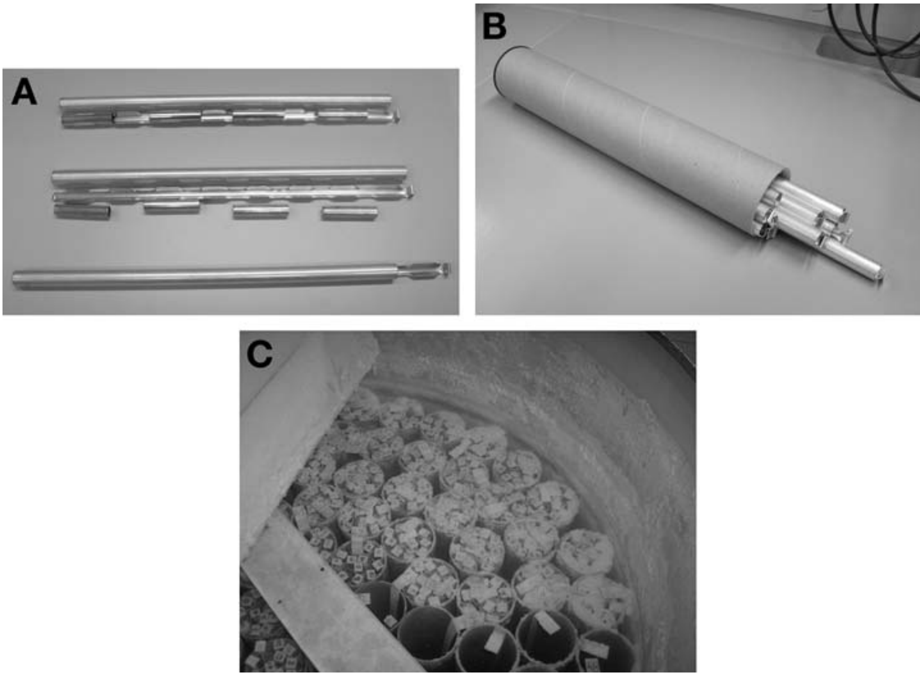

Fig. 5. **(A)** The system used for storing capillaries in the liquid phase in the DSMZ. Bottom in photo the aluminium cane, holders, and sleeve fully assembled. Middle of photo the individual components, holder, cane, and sleeve. Top of photo the holders clipped into the cane, ready to be inserted into the sleeve. **(B)** The aluminium containers held in the cardboard canister. The bottom of the tube is strengthened by a metal plate that has holes drilled in it. **(C)** View into a partially packed tank, showing the empty cardboard canisters (toward the bottom of the photo) and the filled canes and sleeves. Both the canisters and the canes are given a numerical code.

3.3. Drying/Freeze-Drying-Based Preservation/Stabilization Techniques (see Note 3 and Chapter 2)

3.3.1. Freezing and Vacuum-Drying

In this method, a cell suspension in an appropriate suspending fluid is first frozen and then quickly placed under vacuum before the vials have time to thaw. Ice contained in the samples is then removed under vacuum by sublimation.

The method described is that used in the DSMZ and is based on a 10% (w/v) sterile, skimmed milk suspension.

1. Label the outside of the inner vials with suitable details—strain designation, date of preparation, and so on.

2. Either wash the cells from the surface of agar slants using the sterile skimmed milk (20% [w/v]), or resuspend the cell pellet of a centrifuged liquid culture using the skimmed milk.
3. Transfer the skimmed milk suspension into the multistepper and dispense 200 µL of the homogeneous suspension into the stopper vials. Freeze at –20°C.
4. Quickly transfer the frozen material to the freeze-drier and apply the vacuum overnight (primary drying).
5. Once dry, remove the inner vials from the vacuum chamber, cut off the overhanging tops of the cotton stoppers, and place each inner vial in a soda glass tube (**Fig. 4B**).
6. Pack the inner vial into the soda glass tube by firmly packing a layer of about 5 mm of glass wool on top of the vial (**Fig. 4B**).
7. Constrict the ampoules (*see* **Notes 2** and **26**) (**Fig. 6A–D**).
8. Place the ampoules on the freeze-drier, using the "tree" for "secondary drying," and wait until the vacuum has built up again—usually 2–3 h (**Fig. 7A**,**B**).
9. Cut off the ampoules at the narrowest point using the gas torch. Practice is needed to ensure that the glass collapses evenly and forms a uniform seal. The end should be rounded off to give a more stable tip (**Fig. 4B**).
10. Store the ampoules in the dark at reduced temperature.

3.3.2. Vacuum-Drying Using a Predried Plug or Absorbent Material

In this method a relatively large volume of a suitable material (dextran, starch, peptone, skimmed milk) is sterilized and freeze-dried. A small volume of the cell suspension to be dried is then dropped onto this predried plug and then placed under vacuum. It is unclear whether the cell suspension freezes under these conditions. The DSMZ uses a skimmed milk plug, whereas Dando and Bousfield ***(6)*** have described a variation of the centrifugal freeze-drying method that uses a thicker, more absorbent filter paper to soak up the cell suspension, dispensing with the centrifugation step.

1. Dispense the 20% (w/v) skimmed milk solution in 500-µL portions into the stoppered glass vials. Sterilize by autoclaving at 115°C for 13 min.
2. Freeze the sterile skimmed milk-containing vials at –20°C.
3. Quickly transfer the frozen, sterile, skimmed milk to the vacuum chamber of a freeze-drier and apply the vacuum until dry. This may take more than 1 d, depending on the size of the batch. The dried, sterile plugs can be stored covered in suitable containers for some weeks.
4. Label the outside of the inner vials with suitable details—strain designation, date of preparation, and so on.
5. Either wash the cells from the surface of agar slants using 1 mL fresh medium, or resuspend the cell pellet of a centrifuged liquid culture using 1 mL fresh medium.
6. Transfer the cell suspension into the multistepper and dispense 20 µL of the homogeneous suspension into the stopper vials so that the suspension drops onto the middle of the sterile skimmed-milk plug.

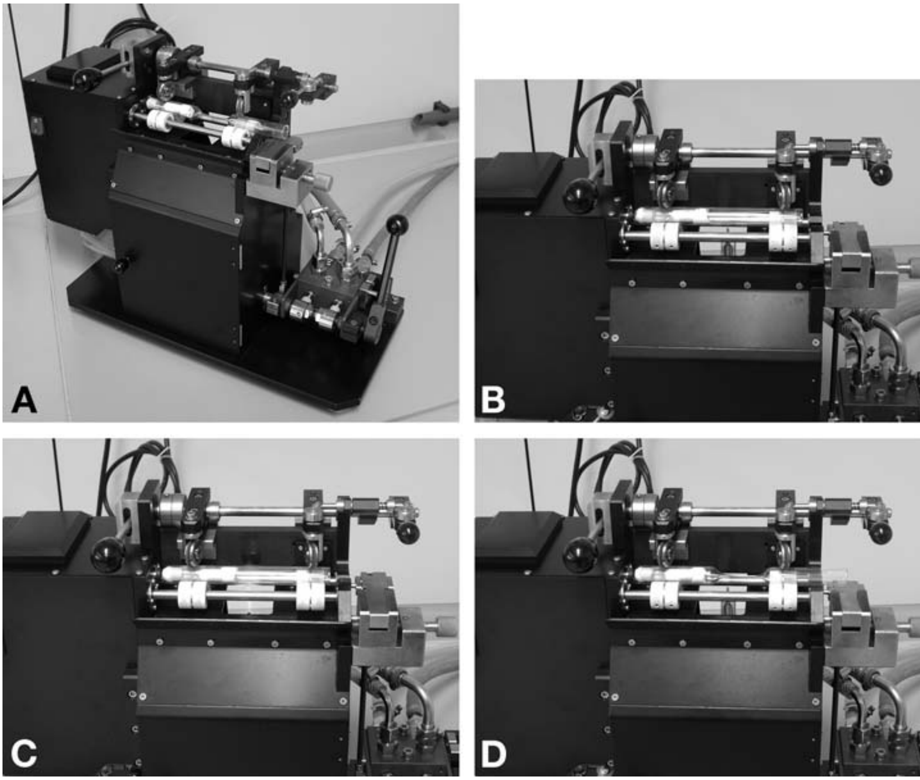

Fig. 6. The process of constricting the DSMZ double vial. **(A)** The constrictor model currently in use in the DSMZ. **(B)** The packed ampoule laying in the bed of the constrictor. The fishtailed burner lies behind the ampoule (middle of photo). **(C)** The packed ampoule laying in the bed of the constrictor, being heated by the fishtailed burner (middle of photo). **(D)** The constricted ampoule laying in the bed of the constrictor. The fishtailed burner has been returned to its position behind the ampoule (middle of photo).

7. Take the inner vials, cut off the overhanging tops of the cotton stoppers, and place each inner vial in a soda glass tube (**Fig. 4B**).
8. Pack the inner vial into the soda glass tube by firmly packing a layer of about 5 mm of glass wool on top of the vial (**Fig. 4B**).
9. Constrict the ampoules (*see* **Notes 2** and **26**) (**Fig. 6A–D**).
10. Place the ampoules on the freeze-drier, using the "tree," and dry overnight (**Fig. 7 A**,**B**).
11. Cut off the ampoules at the narrowest point using the gas torch. Practice is needed to ensure that the glass collapses evenly and forms a uniform seal. The end should be rounded off to give a more stable tip (**Fig. 4B**).
12. Store the ampoules in the dark at reduced temperature.

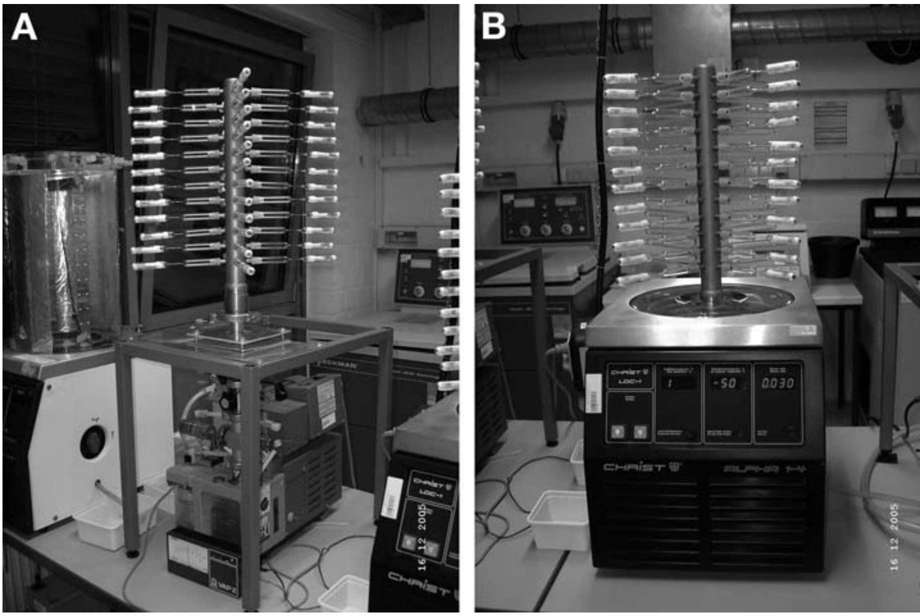

Fig. 7. Two freeze-driers used in the DSMZ. **(A)** An RC4 chemical hybrid pump from Vacuubrand. **(B)** An Alpha 1-4 from Martin Christ.

Dando and Bousfield ***(6)*** have described a variation of this method using thick, absorbent filter paper. This may be substituted for the skimmed-milk plug in this method or used in combination with narrower vials. The authors also used “mist descicans,” rather than growth medium, as the suspending solution.

3.3.3. Centrifugal Freeze-Drying

Using this method a cell suspension, in an appropriate suspending fluid, is placed in a low-speed centrifuge that can be placed under vacuum ***(1,2,6)***. The centrifuge is first switched on and once running the vacuum is then applied. Under these conditions the cell suspension freezes from the application of the vacuum. In the initial stages it is important that the centrifuge remains switched on until the cell suspension is completely frozen, otherwise the cell suspension with froth and bubble. This method is particularly useful for drying organisms that tolerate oxygen for a limited amount of time.

1. Either wash the cells from the surface of agar slants using “Mist desicans,” or resuspend the cell pellet of a centrifuged liquid culture using “Mist desicans.”
2. Transfer the cell suspension into the Pasteur pipet and dispense 100–200 µL of the homogeneous suspension into the bottom of the sterile vials so that the suspension does not contaminate the upper part of the vial.

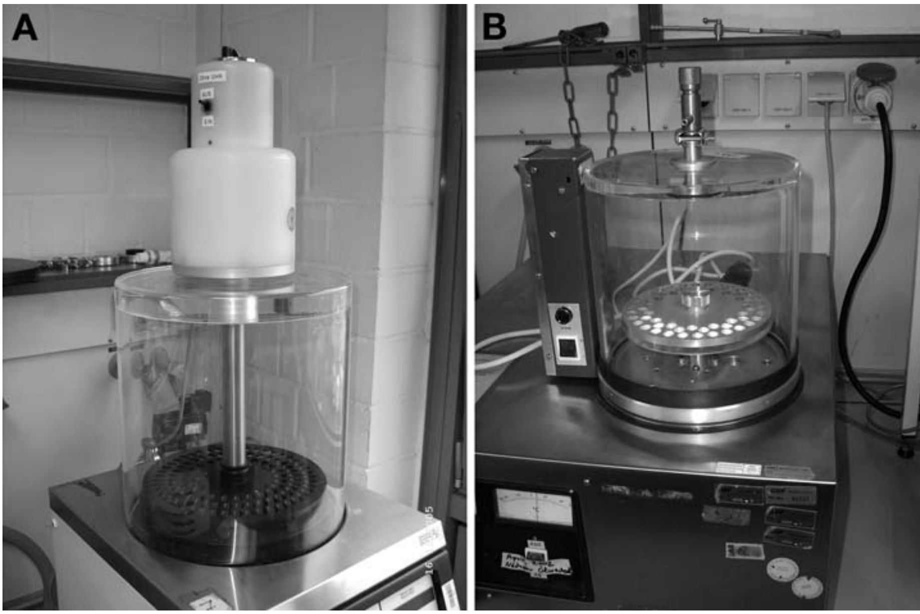

Fig. 8. Two centrifugal drying systems. **(A)** A centrifuge head from Martin Christ, fitted to an Alpha 1–4. **(B)** A centrifuge head made by Edwards High Vacuum, fitted to an Edwards Modulyo freeze-drier. Neither company currently manufactures these parts.

3. Place the stoppered, filled vials in the centrifuge rotor. Start the rotor, and once running at full speed turn on the vacuum. Alternatively, the vials may be covered with close-fitting gauze or cotton caps. “Primary drying” (*see* Chapter 2) continues for a minimum of 3 h (**Fig. 8A**,**B**).
4. Stop the centrifuge and replug the vials with sterile cotton wool.
5. Constrict the ampoules (*see* **Notes 2** and **26**) (*see* **Fig. 6A–D**).
6. Place the ampoules on the freeze-drier, using the “tree” for secondary.
7. Once secondary drying is complete cut off the ampoules at the narrowest point using the gas torch. Practice is needed to ensure that the glass collapses evenly and forms a uniform seal. The end should be rounded off to give a more stable tip (**Fig. 4A**).
8. Store the ampoules in the dark at reduced temperature.

Hippe *(5)* described a variation of this method for anaerobes (using a suspending fluid containing 1 mg/mL amorphous iron [II] sulfide) and the DSMZ “double-vial” method whereby the inner vial is placed in a vacuum centrifuge and later packed into the outer vial. The vacuum is released by flushing the vacuum chamber with nitrogen.

3.4. Quality Control of Frozen and Dried Cultures

The viability of all strains stored in the DSMZ is checked by comparing the viability of the cell suspension used to prepare the frozen or vacuum-dried

stocks with that of the stocks after they have been frozen or vacuum dried. The method is a simple 10-fold dilution series viable count.

1. Viability counts before storage: take the same volume of cell suspension used in preparing the vials/ampoules capillaries (or a known volume of the glycerol/strain mixture or a single bead) and prepare a 10-fold dilution series in the liquid media. Aerobes may be plated out by dropping one drop from a sterile Pasteur pipet from each tube onto the same medium solidified with agar. The drop may be gently distributed using a sterile loop or sterile glass rod. Typically an agar plate may be divided into either three or six equal sectors and one drop from the Pasteur is placed in that sector, beginning at the lowest dilution. Incubate the strain at the appropriate temperature until growth occurs.
2. Viability counts after storage: this requires that a frozen vial/capillary or vacuum-dried ampoule is opened.
   a. Screw-cap vials may be transported to the lab in specially prepared containers that have holes into which the vials may be placed. Alternatively, wax is poured into a suitably stable container and a hole is drilled large enough to take the vial being used. The blocks/containers are routinely stored in the freezer. Open the vials and either remove a known portion of the material or a single bead. Proceed as in **step 1**.
   b. Vacuum-sealed ampoules can be opened in one of two ways, depending on their diameter. The smaller diameter (7–8 mm "single" ampoules) are scored with a diamond or glass cutter in the middle of the region where the cotton plug is located. Sterilize the outside by wrapping it in an alcohol-soaked cloth. Carefully break open the vial, making sure that the cotton plug remains in the section where the dried cell material is located. The larger "double vials" (such as those supplied by the DSMZ) are opened by placing the tip of the vial in a Bunsen flame. The vial is removed from the flame and a drop of distilled water placed on the glass to crack it. Wear goggles if working outside of a safety cabinet. The tip should crack but otherwise remain intact. Hold the tip over a suitable container (beaker) and gently tap the end with a hard object, such as the end of a set of forceps (do not use a hammer). The end should fall off. Remove the upper glass wool plug with care, using forceps, and dispose of it carefully (the glass wool may be a health hazard). Remove the inner vial.
3. Hydrate the contents of the vials using 0.2–0.5 mL freshly prepared growth medium. Oxygen sensitive strains should be handled in a way that reduces exposure to oxygen (e.g., Hungate technique). In the case of predried plugs they may take 15–30 min to fully rehydrate. Remove all of the contents of the vial and inoculate the first tube of liquid medium. Prepare a dilution series as in **step 1**.
4. Capillaries are removed from their storage position and immersed in a small water bath. Pyschrophiles may need to be thawed in iced water. Using forceps, remove the capillary from the water bath and place it on dry, absorbent paper. Dry the capillary. Using a sharp, fine diamond lightly score the capillary at both ends (about 5 mm from each end). Holding the capillary in self-closing forceps dip the ends of

the capillary in 100% ethanol so that the score mark is immersed, remove the capillary from the ethanol, and ignite the end with a small, cool flame (e.g., a lighter). Sterilize both ends in the same way. Using flamed forceps break off the ends of the capillary by breaking *away* from the score mark. Be careful not to contaminate the open ends. The contents of the capillary may be removed with either a suitably sized sterile syringe and needle (that fits into the capillary), or using finely drawn out Pasteur pipets. Anaerobes are best removed using a sterile syringe and needle, which has been flushed with sterile, oxygen-free nitrogen. Inoculate the first tube of liquid medium and prepare a dilution series as in **step 1**.

3.5. Authenticity/Identity Checks

There is no standard method for confirming the authenticity/identity of a culture. The methods used for a collection of strains of different species will differ from those used to distinguish a collection of mutant strains of the same species. In some cases, the organisms may not even be fully characterized and the sole goal is to make sure that strains are not swapped or contaminated. The methods available to a national culture collection may also differ to those available to a research laboratory.

4. Notes

1. The adapter used in the author's laboratory is made from the top of a 1-mL pipet, which has a wide top to take a cotton plug (inside diameter approx 5.3 mm) (**Fig. 2C**). Using a very hot flame (a glass blower's torch would be ideal) heat the pipet about 50–60 mm from the top end. When the glass softens gently pull the two ends apart to give a conical constriction. Allow to cool and separate the two halves of the pipet with a glass cutter. Round off the sharp ends of the shorter (top) end in a hot flame. Three to five millimeters above the region where the cotton plug narrows to join the main bore of the pipet, score the glass with a glass cutter and break off the section with the wider bore. Round off the sharp ends of the remaining section in a hot flame. Insert a piece of cotton wool into the bore so that it forms a plug midway between the two ends of the glass adapter. Insert a short piece (5–6 mm, 3-mm o.d., 0.8-mm wall thickness) of silicon rubber tubing (3-mm o.d., 0.8-mm wall thickness) into the bore of the pipet where the wider bore constricts into the narrower (main bore of the pipet). Wetting the silicon tube with water may help the tubing to slide into place. The combination of the constriction in the glass bore and the silicon rubber tubing forms a seal when the end of the capillary is inserted into the adapter (similar in principle to the way most pipetting aids function). The pipetting aids are sterilized by placing them in short, glass, screw-capped tubes (16×100 mm). A different, sterile adapter is used for each strain to be handled.
2. Vacuum drying in glass; constriction and sealing of the ampoules: when drying strains under vacuum there are two important steps that are crucial in preparing the ampoules. The first is to create a constriction (narrowing of the diameter) of the

ampoule, without which it would be impossible to seal the glass under vacuum. The second important stage is sealing the ampoule under vacuum.

Many culture collections use simple glass ampoules, which are 7 × 100 mm (wall thickness approx 1 mm). In the past, Edwards used to supply a machine (a constrictor) to help produce a uniform narrowing of the ampoule prior to the final evacuation of the vial. This machine is no longer available and several culture collections have had their own machines custom made. It is possible to produce a constriction in the vials by turning the ampoules in a fine hot flame. The constriction being produced by gentle pulling with the ampoule is rotated. This requires some skill and practice and is often the limiting factor is determining whether one embarks on the task of freeze drying a collection of strains.

3. General considerations on the type of freeze-drier to be used: over the decades various freeze-driers have been used for the vacuum-drying of cultures. Some publications centers on a particular model used in a particular collection, but few articles deal with some of the general principles. The following information may be helpful:
   a. The size of the freeze-drying unit should fit the needs of the laboratory. In some cases a single, large freeze-drier may not be the best option when handling many different strains with a variety of growth requirements and where different methods of drying are to be used. In many cases, simple apparatus may suffice if properly designed and implemented (**Fig. 7A**,**B**).
   b. Under normal vacuum-drying conditions a relatively high vacuum should be obtained, which means that oil rotary vacuum pumps are essential.
   c. During the course of vacuum-drying water must be removed from the sample and should not be allowed to collect in the oil of the rotary pump. There are a number of ways to prevent water collecting in the pump oil.
      i. Some systems used a phosphorus pentoxide trap between the vials and the vacuum pump. The old Edwards 5PS freeze-drying machine (that may still be in use) was such an example. Phosphorus pentoxide is, of course toxic, will cause burns, and must be handled with care.
      ii. Most modern freeze-driers use a refrigerated cold trap to remove the water by freezing in a chamber that is placed between the vials and the vacuum pump. Such systems were the Edwards Modulyo series and the machines currently produced by Martin Christ (**Fig. 7B**).
      iii. Experiments in the DSMZ have shown that pumps made by Vacuubrand (Wertheim, Germany) for use in chemical laboratories, such as the RC4 (**Fig. 7A**) and RC5, chemical hybrid pumps (both have been replaced by the RC6) can be used without the need for either phosphorus pentoxide or a cold trap. Essentially these pumps are a rotary vacuum pump, to the exhaust of which is added a membrane vacuum pump. Oil pumps get hot when running, which means that if the oil is put under additional vacuum (with a membrane pump) then any water collecting in the oil will also be removed. This type of pump also has the advantage that the large "dead space" needed by either the phosphorus pentoxide reservoir or cold trap is not required.

iv. Finding combined centrifugal driers of the type produced by Edwards (5PS or the centrifuge head for the Modulyo) or by Martin Christ in the past is becoming difficult (**Fig. 8A**,**B**). Photographs of the Edwards Modulyo centrifuge and the unit that used to be produced by Martin Christ are included for reference. Both use a lightweight plate in which suitably sized angled (it is important that the holes are at an angle) holes have been drilled. The plate is spun directly by a suitable motor. In the case of the Edwards unit, the motor is below the centrifuge plate (and fits into the cold trap chamber being fixed with screws), whereas the Martin Christ unit is driven by a motor placed on top of the vacuum chamber. High rotation speeds are not needed; they should be sufficient to prevent foaming and frothing during the initial stages of centrifugal drying. The alternative is to use a separate vacuum centrifuge.

4. These organisms are best grown in liquid culture, but once the cells are harvested, and there is no indication that oxygen may be toxic, they may be handled as normal aerobes in all subsequent steps.
5. Some organisms may be particularly fragile (e.g., many members of the family Halobacteriaceae) and great care should be taken not to lyse such cells.
6. Because of the different methods that can be used for either freezing or drying cells, the suspending medium may be either fresh growth medium, fresh growth medium containing cryoprotectants (for freezing only), skimmed milk, or other suitable protective agents. It should be noted that some experimentation may be needed with the organisms concerned simply because there is no single universal methods that will work well for all prokaryotes.
7. Should the strains be grown in a medium that contains particulate matter (e.g., sulfur or cellulose) it is best to grow the strain in a separate vessel, allow the particulate matter to settle out by standing the culture for about 30 min, and then transfer the supernatant to the centrifuge tubes.
8. Some strains may be difficult to harvest if they produce large amounts of extracellular polysaccharide or similar material, and it may be necessary to increase the length of time in the centrifuge or to increase the speed of rotation. However, the glass centrifuge tubes should never be used in centrifuges where the speed of rotation may be increased to 10,000 rpm or more.
9. Under normal conditions the sealed glass tubes will prevent the formation of aerosols. However, when working with organisms of risk group 2 it is a wise precaution to use swinging buckets that are themselves contained in a rotor that may be sealed.
10. If the strains need to be transferred from the original cultivation vessel to the centrifuge tube, the gas mixture in the centrifuge tube must be the same as in the culture vessel.
11. The following cover a number of special cases where the use of the wrong technique can result in the organisms not surviving:
    a. Obligate acidophiles, such as members of the genera *Thermoplasma* or *Sulfolobus*, should be grown in liquid media at low pH. Prior to harvesting the

cultures by centrifugation, cool the cultures to room temperature and an excess of sterile, powdered calcium carbonate should be added to the liquid cultures. This has the effect of increasing the pH to about 6.0. The excess calcium carbonate should be allowed to settle out and the supernatant collected and cells harvested by centrifugation. If the medium is not neutralized in this fashion, members of these two genera will not survive freezing or vacuum-drying.

12. When working with obligate psychrophiles it may be necessary to precool all media and, occasionally, all glassware. Some psychrophiles that could not be vacuum-dried at room temperature, have been successfully dried using a freeze-drier operating in a cold room at 8°C.
13. Members of the family Halobacteriaceae require high salt concentrations. If suspended in low-salt solutions ("mist desiccans," or skimmed milk) they will lyse. These organisms also do not seem to tolerate freezing and drying ***(9)***.
14. Members of the genus *Thermus* may be grown on a variety of media, but they do not always freeze-dry successfully if resuspended in their fresh growth medium. Most strains seem to prefer to be resuspended in Oxoid nutrient broth when the DSMZ predried skimmed milk plug is used.
15. Dando and Bousfield ***(6)*** indicate that methylotrophs do not store well for long periods when glycerol is used as a cryoprotectant. DMSO may be more suitable (Tindall, unpublished).
16. The original version used at the University of Leicester was "homemade," with glass/ceramic beads being purchased from a hobby shop, washed and cleaned before being sterilized and used. However, a number of commercial systems are also available that provide everything needed in kit form (e.g., Cryobank from Mast, CryoLine products from Nunc). An important factor is that the cell suspension either coats the bead, or the cell suspension is contained in the hole in the bead. Theoretically, this makes removing individual beads easier. However, every time a bead is removed there is always a risk (however small) of the stored material being contaminated.
17. Feltham et al. ***(7)*** and Jones et al. ***(8)*** recommend the use of Oxoid nutrient broth containing 15% (v/v) glycerol with work at the University of Leicester centered on medically important organisms.
18. Storage of anaerobic strains on glass/ceramic beads: Jones et al. ***(8)*** have indicated that glass beads may also be used to store anaerobes. However, it should be remembered that exposure of strains to oxygen on the large surface area of the beads may reduce the viability of the strains.
19. There are a number of risks associated with storage or cell suspensions in glass or plastic vials using liquid nitrogen. If screw-capped glass vials are used, the sealing rubber gasket will become brittle and hard at the low temperatures in the liquid nitrogen tank. This will cause the seals to leak and they will fill with cold nitrogen gas (if stored above the gas phase), or will fill with liquid nitrogen if stored in the liquid phase. On removal from the liquid nitrogen tank the unopened vial will explode violently. The alternative is to use plastic vials. However, they too have a seal, which may, if not handled properly, also leak. Leaks may be a potential source of contamination. However, exploding vials are also a potential health

risk, as well as leading to the loss of valuable biological material. Wherever possible the methods used in the DSMZ for the storage of strains in liquid nitrogen avoids the use of glass or plastic vials. Instead a glass capillary system has been developed ***(5)***.

20. The question of whether to use glass or plastic from freezing strains in the temperature range from –20 to –80°C is not given enough attention. Glass has a number of advantages, including the fact that it can be easily sterilized without distortion. Plastic containers, such as Eppendorf caps or similar products with a flip lid, are to be avoided at all costs. They may be suitable for storing small portions of chemical reagents, but are *not* suitable for storing strains. The flip-lid action is probably the best way of contaminating the contents.

    When storing strains using liquid nitrogen, screw-cap glass vials are problematic because it is very difficult to ensure a good seal, and if nitrogen enters the vial on removal from the tank they will explode violently. Several plastic vials are available, specifically designed for use in liquid nitrogen tanks, although it is generally recommended that they be used in the gas phase. Again, improperly sealed plastic vials will explode violently when removed from the liquid nitrogen tank. In this respect the capillary system developed in the DSMZ is one of the best and safest methods of storing strains in liquid nitrogen.
21. The advantages of capillaries vs plastic cryovials include: a single capillary is used to inoculate the medium and other capillaries prepared in parallel are left unopened, reducing the risk of subsequent contamination of the stored material to zero; the samples are sealed in glass, which is largely impermeable to gases, making the system ideal for the long-term storage of strains that are sensitive to oxygen; the capillaries are dense enough to be stored in the liquid nitrogen phase—they also do not leak.
22. The method used is the same as that given in **step 26**, except that 15% (v/v) glycerol should be mixed with a suitable, freshly prepared anaerobic medium, preferably the same medium as the growth medium.
23. Experience usually indicates when capillaries are not properly sealed. However, it is a wise precaution (and certainly to be recommended for beginners) to check that the ends of the capillaries are properly sealed and formed by examining them using a binocular dissecting microscope. The first end sealed may be slightly bulbous and rounded, whereas the other end is slightly constricted.
24. In the DSMZ all strains are stored in the liquid phase of liquid nitrogen. The storage containers used in the DSMZ are aluminium tubes that are clipped onto “canes” (that can normally hold appropriately sized plastic vials) (**Fig. 5A**). Depending on the depth of the tank, four or five containers may be clipped to the canes, each being filled with up to 40 capillaries. The whole is then placed in an aluminium sleeve. Up to 12 such sleeves and canes are contained in cardboard inner canisters (**Fig. 5B**). A large 350-L liquid nitrogen tank will hold approx 100 of these cardboard inner containers (**Fig. 5C**). If each aluminium tube on a “cane” contains 1 strain, then a single 350-L liquid nitrogen tank will hold 4800–6000 strains. In addition, capillaries may also be color coded by placing them in closely fitting plastic sleeves. If only 10 capillaries per strain are prepared and each strain color coded, then up to

three strains per aluminium tube may be stored. This increases the capacity of a single 350-L liquid nitrogen tank to 14,400–18,000 strains.

25. At the final stage nitrogen is the gas of choice; using nitrogen:carbon dioxide mixtures causes the sealed capillaries to crack when frozen.
26. The DSMZ uses a double-vial system. The inner vial is 11 × 43.5 mm and is contained within a soda glass tube, initially 14.5 × 140 mm. It is almost impossible to constrict these by hand. The constrictor model that was constructed in cooperation with the DSMZ is illustrated in the accompanying photos (**Fig. 6A**). The combined inner vial and outer tube are placed in the bed of the machine, and rotates on the two pairs of ceramic wheels (**Fig. 6B**). A gas–air fishtail burner initially lies behind the tube and runs on a small flame. A lever is pulled forward and brings the burner into position directly below the tube, at the same time allowing the gas–air flow to reach their set maximum (**Fig. 6C**). As the tube rotates in the flame and softens, the two upper angled guide wheels begin to draw the tube apart. Experience is needed to know when the glass is hot/soft enough to be properly constricted. The burner lever is pushed back, the flame is reduced in intensity, and is moved away from the hot tube. The upper lever to the right is then moved to the right, increasing the angle of the right wheel, and rapidly draws the outer tube to the right, forming a narrow constriction (**Fig. 6D**). Care must be taken that the constriction is not too wide, nor so narrow that it is closed. The width of the bore of the constriction and the thickness of the glass all contribute to the stability of the final ampoules when they are sealed.

Acknowledgments

I would like to thank the various members of the scientific staff and technicians of the DSMZ, past and present, who have contributed to the development and routine use of some of these methods in the DSMZ. I would also like to thank those scientists in other institutions that have either passed on their knowledge in the written word or verbally. This work underlies the maintenance of our microbiological scientific heritage, which can be passed on to the next generation in the knowledge that they are working with the same strains as their predecessors.

References

1. Rudge, R. H. (1991) Maintenance of bacteria by freeze-drying. In: *Maintenance of Microorganisms and Cell Cultures*, *2nd ed.*, (Kirsop, B. E. and Doyle, A., eds.), Academic Press, London, UK, pp. 31–44.
2. Lapage, S. P. and Redway, K. F. (1974) *Preservation of Bacteria With Notes on Other Microorganisms.* Public Health Laboratory Service Monograph Series 7 London.
3. Kirsop, B. E. and Doyle, A. (1991) *Maintenance of Microorganisms and Cell Cultures*, *2nd ed.*, Academic Press, London, UK.
4. Impey, C. S. and Phillips, B. A. (1991) Maintenance of anaerobes. In: *Maintenance of Microorganisms and Cell Cultures*, *2nd ed.*, (Kirsop, B. E. and Doyle, A., eds.), Academic Press, London, UK, pp. 71–80.

5. Hippe, H. H. (1991) Maintenance of methanogenic bacteria. In: *Maintenance of Microorganisms and Cell Cultures*, *2nd ed.*, (Kirsop, B. E. and Doyle, A., eds.), Academic Press, London, UK, pp. 101–114.
6. Dando, T. R. and Bousfield, I. J. (1991) Maintenance of industrial and marine bacteria. In: *Maintenance of Microorganisms and Cell Cultures*, *2nd ed*., (Kirsop, B. E. and Doyle, A., eds.), Academic Press, London, UK, pp. 57–64.
7. Feltham, R. K. A., Power, A. K., Pell, P. A., and Sneath, P. H. A. (1978) A simple method for the storage of bacteria at –70°C. *J. Appl. Bacteriol*. **44,** 313–316.
8. Jones, D., Pell, P. A., and Sneath, P. H. A. (1991) Maintenance of bacteria on glass beads at –60°C to –76°C. In: *Maintenance of Microorganisms and Cell Cultures*, *2nd ed.,* (Kirsop, B. E. and Doyle, A., eds.), Academic Press, London, UK, pp. 45–50.
9. Tindall, B. J. (1991) Cultivation and preservation of members of the family Halobacteriaceae. *World J. Microbiol. Biotechnol*. **7,** 95–98.